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Alphavirus-based Vaccines Encoding Nonstructural Proteins of Hepatitis C Virus Induce Robust and Protective T-cell Responses

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An absolute prerequisite for a therapeutic vaccine against hepatitis C virus (HCV) infection is the potency to induce HCV-specific vigorous and broad-spectrum T-cell responses. Here, we generated three HCV vaccines based on a recombinant Semliki Forest virus (rSFV) vector expressing all- or a part of the conserved nonstructural proteins (nsPs) of HCV. We demonstrated that an rSFV vector was able to encode a transgene as large as 6.1 kb without affecting its vaccine immunogenicity. Prime-boost immunizations of mice with rSFV expressing all nsPs induced strong and long-lasting NS3-specific $CD8^+$ T-cell responses. The strength and functional heterogeneity of the T-cell response was similar to that induced with rSFV expressing only NS3/4A. Furthermore this leads to a significant growth delay and negative selection of HCV-expressing EL4 tumors in an *in vivo* mouse model. In general, as broad-spectrum T-cell responses are only seen in patients with resolved HCV infection, this rSFV-based vector, which expresses all nsPs, inducing robust T-cell activity has a potential for the treatment of HCV infections.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of liver disease and the primary cause for liver transplantation in the western world. The World Health Organization estimated that ~150 million people worldwide, *i.e.*, around 2.5% of the world population, are chronically infected with HCV. Sixty to 70% of these patients develop chronic liver disease such as liver fibrosis and cirrhosis and 1–5% of these patients develop liver cancer within 10–40 years after infection.[1] The former standard of care treatment, a combination of antiviral agents, pegylated interferon-α and ribavirin, achieved sustained viral response in less than 50% of the patients with chronic HCV (CHC) infected with HCV genotype 1.[2] Currently, in some countries, this standard of care treatment is being combined with the novel HCV protease inhibitors boceprevir and telaprevir, resulting in a sustained viral response of ~70% in patients infected with HCV genotype 1.[3,4] Yet these drugs are not widely available, the drugs are not always tolerated and not all genotypes respond equally to interferon and the antiviral drugs. In contrast to vaccines against hepatitis A and B virus, there is no prophylactic HCV vaccine available, amongst other reasons due to the high variability of the structural proteins of the virus. Therapeutic vaccines, aimed at inducing T-cell responses against the more conserved proteins of the virus are therefore urgently needed. These vaccines can possibly be used as single treatment modalities or can be combined with standard antiviral treatments.

HCV-specific adaptive cellular immunity plays an essential role to control HCV infection, particularly in the induction of functional HCV-specific T cells[5] as also exemplified by the following observations. The presence of NS3/4A-specific $CD8^+$ T cells is positively correlated with a sustained viral response.[6–8] Patients who develop CHC have a narrow-spectrum and low number of HCV-specific T cells in both circulating blood and liver.[5] A low ratio of the HCV-specific $CD8^+$ T cells to the nonspecific $CD8^+$ T cells in infected liver leads to an ineffective clearance of virus and a nonspecific inflammation.[9] Next, dysfunctional HCV-specific T cells with reduced IL-2 secretion and cytotoxic activities are being observed in patients with CHC,[10,11] and surprisingly, also in patients who recovered from chronic HCV infection by IFN/ribavirin treatment.[12] Moreover, the longer the duration of the exposure of HCV antigen, the deeper the level of T-cell exhaustion.[12] Thus, immunotherapeutic approaches against HCV, should not only induce *de novo* HCV-specific T-cells production but also restore T-cell function.

Several immunotherapeutic approaches are being developed to induce HCV-specific immune responses.[13] Among those approaches, viral vectors induce the most robust immune response in both preclinical and clinical settings. In this study, recombinant Semliki Forest virus (rSFV) vector, which induces strong and long-lasting antigen-specific response,[14] was used to develop a therapeutic vaccine against HCV. The nonstructural proteins (nsPs) of HCV have been identified as promising vaccine targets due to the fact that they are genetically conserved, essential for viral replication and most importantly, immunogenic. In an effort to improve immune responses against the nsPs of HCV, we generated three rSFV constructs encoding either the entire nsPs of

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HCV (1. NS2′-5B′) or parts of these proteins of HCV (2. NS3/4A and 3. NS5A/B′). The *in vivo* efficacy of these rSFV-based vaccines was determined in naïve and tumor-bearing mice.

RESULTS

Characterization of rSFV encoding the entire or the part of HCV nsPs

Aiming to induce immune responses against the entire or part of the HCV nsPs, three rSFV expressing (i) NS2′-5B′, (ii) NS3/4A, and (iii) NS5A/B′ proteins of HCV, were designed and produced (**Figure 1a**). Production and stability of the HCV nsPs synthesized by rSFV infected BHK-21 cells were determined by ^{35}S-methionine pulse labeling (**Figure 1b**). Incubation with rSFVeNS3/4A induced production of the NS3/4A fusion protein (75.9 kDa) and the NS3 protein (70 kDa) by BHK-21 cells. On the other hand, cells incubated with rSFVeNS2′-5B′ synthetized five distinct proteins, corresponding to the NS2/3/4A fusion protein (86.9 kDa), the NS2/3 fusion protein (81 kDa), the NS5B protein (60.8 kDa), the NS5A protein (49 kDa), and the NS4B protein (28.7 kDa). Cells incubated with rSFVeNS5A/B′ produced one NS5A/B fusion protein (109.8 kDa). Cells incubated with control rSFVe or buffer were negative controls. Proteins expression was also determined by western blotting stained with anti-NS3 and anti-NS5A antibodies (data not shown). The newly constructed rSFVs induced abundant expression of HCV nsPs which were stably expressed till 22 hours after incubation with rSFVs.

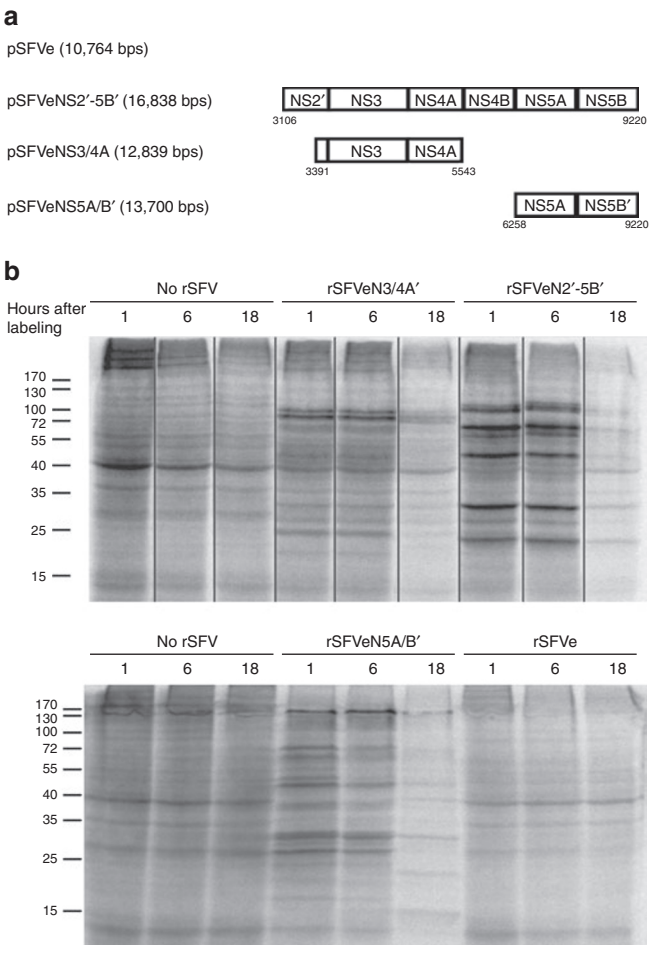

Figure 1 Stable expression of hepatitis C virus (HCV) nsPs *in vitro*. (**a**) Size of plasmid Semliki Forest virus (SFV), number is the nucleotide position in the plasmid DNA containing full genome of HCV 1a (H/FL). (**b**) BHK-21 cells were incubated with rSFVeNS2′-5B′, rSFVeNS3/4A, rSFVeNS5A/B′, or rSFVe. "No rSFV" was negative control cultured in medium only. Cells were pulsed with ^{35}S-methionine for 1 hour at 6 hours after addition of rSFV and were further cultured for 1, 6, or 18 hours. Radioactively labeled proteins were revealed by autoradiography after 12% SDS-PAGE. The molecular weight of each nonstructural protein is as following: NS2′: 11 kDa, NS3: 70 kDa, NS4A: 5.9 kDa, NS4B: 28.7 kDa, NS5A: 49 kDa, NS5B′: 60.8 kDa. Vertical lines on the upper image show where separate lanes from the same gel were juxtaposed for better comparison with the lower image. Data represent two independent experiments.

Frequencies and phenotypes of NS3-specific T cells induced by rSFV immunizations

Next, to verify the immunogenicity of these newly constructed rSFV vaccines *in vivo*, mice were intramuscularly primed and boosted with 5×10^6 purified rSFV or buffer (phosphate-buffered saline (PBS)) with a 2-week interval and the *in vivo* induction of NS3-specific T-cells response was evaluated. Ten days after the last immunizations, NS3-specific CD8$^+$ cells were identified by GAVQNEVTL-dextramer and the phenotype of these cells was studied. The peptide GAVQNEVTL is derived from HCV NS3 and has been identified as a potent cytotoxic T lymphocytes (CTL) epitope presented by the major histocompatibility complex (MHC) class I molecule H-2D^b of C57BL/6 mice.[15] Both rSFVeNS2′-5B′ and rSFVeNS3/4A immunizations induced potent NS3-specific CD8$^+$ T-cell responses, yet mice immunized with rSFV encoding only NS3/4A had higher frequencies than mice immunized with rSFV encoding the entire HCV nsPs (rSFVeNS2′-5B′: 2.2% ± 0.4% versus rSFVeNS3/4A: 5.5% ± 0.4%, $P < 0.05$) (**Figure 2a**). The NS3-specific CD8$^+$ T cells were classified into three subsets, corresponding (i) central memory T cells (T_{CM}, CD44$^+$CD62L$^+$CD127$^+$), (ii) effector memory T cells (T_{EM}, CD44$^+$CD62L$^-$CD127$^+$), and (iii) effector T cells (T_{eff}, CD44$^+$CD62L$^-$CD127$^-$). The total number of each T-cell subset in the spleen is shown (**Figure 2b**). The increased number of the total NS3-specific CD8$^+$ T cells in mice immunized with rSFVeNS3/4A (**Figure 2a**) was mainly due to the increase in the number of T_{EM} cells and T_{eff} cells ($P < 0.05$). Immunization with rSFV encoding either the entire or the part of HCV nsPs induced NS3-specific responses with similar frequencies of T_{CM} cells.

Effector function of HCV-specific CD8$^+$ cells

In order to investigate the spectrum of the HCV-specific response, spleen cells from rSFV-immunized mice were stimulated with stimulants containing the entire or part of the HCV nsPs and the effector functions of HCV-specific CD8$^+$ T cells were determined. The stimulants were Hepa1-6^V cells, Hepa1-6^V-NS2′-5B′ cells, or the HCV synthetic peptides, GAVQNEVTL or ILDSFDPL. Hepa1-6^V-NS2′-5B′ cells are murine hepatoma cells expressing HCV NS2′-5B′ protein as well as a yellow fluorescence reporter protein, VENUS (abbreviated to ^{V}) and Hepa1-6^V cells are mock transduced cells expressing VENUS protein only (**Supplementary Figure S1**). The peptide ILDSFDPL has been recently identified as a HCV NS5A CTL epitope presented by the MHC class I molecule, H-2K^b of C57BL/6 mice.[16]

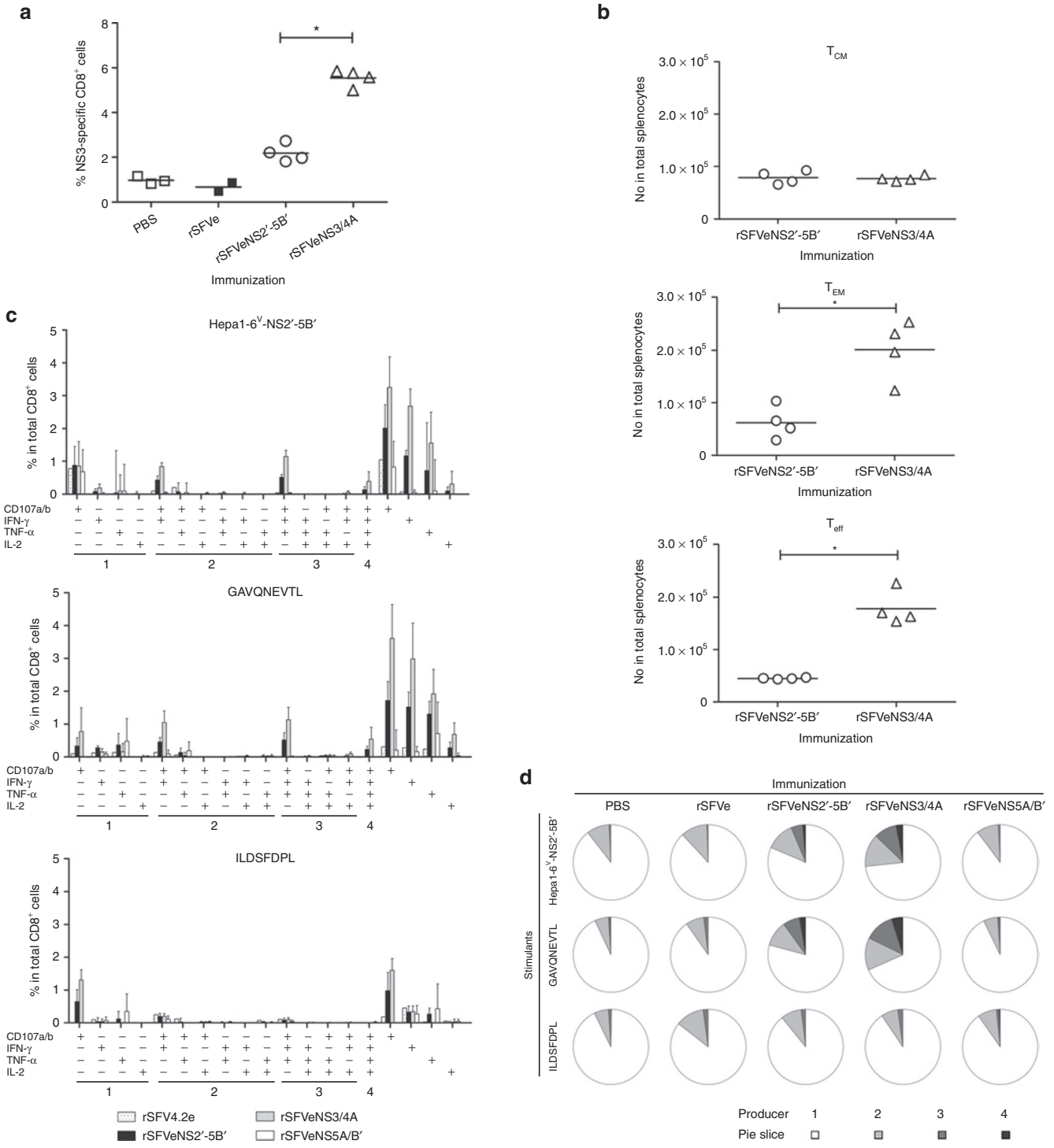

Figure 2 Induction of polyfunctional hepatitis C virus (HCV)-specific CD8$^+$ cells by recombinant Semliki Forest virus (rSFV) immunizations. Mice were primed and boosted intramuscularly with 5×10^6 rSFV or phosphate-buffered saline (PBS) with a 2-week interval. Ten days after the last immunization, mice were sacrificed and splenocytes were isolated for phenotypic analysis using flow cytometry. (**a**) NS3-specific CD8$^+$ cells are shown as a percentage of total CD8$^+$ cell population. Data represent two independent experiments showing similar result (n = 2–4). (**b**) Absolute numbers of T_{CM} (CD44$^+$CD62L$^+$CD127$^+$), T_{EM} (CD44$^+$CD62L$^-$CD127$^+$), and T_{eff} (CD44$^+$CD62L$^-$CD127$^-$) cells of the total NS3$^+$CD8$^+$CD44$^+$ cell population in the spleen. (**c**) Splenocytes were stimulated with Hepa1-6^V-NS2′-5B′ cells, GAVQNEVTL or ILDSFDPL peptides for 4 hours and subjected to surface and intracellular cytokines staining. The frequencies of each T-cell subset in the CD8$^+$ cell population are shown. Background (PBS immunized) subtraction was applied. (**d**) Pie charts present the mean frequencies of T-cell subsets. (**c**,**d**) 1, single producers; 2, double producers; 3, triple producers; 4, quadruple producers. Data represent the (**a**,**b**,**d**) mean and (**c**) mean + SD with n = 2–4. *$P < 0.05$.

The binding affinities of both synthetic peptides were confirmed on RMA-S cells. The GAVQNEVTL peptide bound to H-2D^b at as low as 0.3 µmol/l, while the ILDSFDPL peptide bound to H-2K^b at higher concentration (>30 µmol/l) (**Supplementary Figure S2**). *In vitro* stimulation with the Hepa1-6^V-NS2′-5B′ cells or GAVQNEVTL peptide induced degranulation (CD107a/b$^+$) and secretion of multiple cytokines by CD8$^+$ cells from mice immunized with rSFVeNS2′-5B′ and rSFVeNS3/4A (**Figure 2c,d**). However, very weak response was detected in mice immunized with rSFVeNS5A/B′. Stimulation with neither Hepa1-6^V cells (**Supplementary Figure S3**) nor ILDSFDPL induced multifunctional CD8$^+$ T cells (**Figure 2c,d**). Of note, immunization with rSFVeNS2′-5B′ and rSFVeNS3/4A increased the frequencies of polyfunctional CD8$^+$ T cell subsets that produced more than 1 cytokine (*e.g.*, CD107a/b$^+$IFN-γ$^+$TNF-α$^+$ and CD107a/b$^+$IFN-γ$^+$TNF-α$^+$IL-2$^+$ populations) to similar level upon specific stimulations (**Figure 2d**). Lower numbers of NS3-specific CD8$^+$ cells were detected in mice immunized with rSFV encoding the entire HCV nsPs, the effector functions of these HCV-specific CD8$^+$ cells were similar to mice immunized with rSFV expressing NS3/4A only.

Induction of HCV-specific CTL with rSFV immunizations

The most important criterion for functional effector T cells is the ability to lyze their target cells, which, in this study, are the HCV-infected cells. Therefore, the cytolytic activity of HCV-specific CTLs was investigated *in vitro* (**Figure 3b–d**) and *in vivo* (**Figure 3e**). CTL activity was determined 10 days after the last immunization. To detect cytotoxicity *in vitro*, spleen cells isolated from immunized mice were restimulated with the Hepa1-6^V-NS2′-5B′ cells for 7 days. The number of NS3-specific CD8$^+$ T cells induced after 7-day restimulation was higher in mice immunized with rSFVeNS3/4A (58.2% ± 15.8%) than in mice immunized with rSFVeNS2′-5B′ (27.6% ± 7.0%) ($P < 0.05$) (**Figure 3a**). This correlated with the higher number of precursor cells in mice immunized with rSFVeNS3/4A (**Figure 2a**). Restimulated CTLs from mice immunized with rSFVeNS2′-5B′ or rSFVeNS3/4A lysed Hepa1-6^V-NS2′-5B′ cells (**Figure 3b**) and GAVQNEVTL-pulsed EL4 cells (**Figure 3c**) to a similar extent. In general, there was a higher cytotoxic activity against the GAVQNEVTL-pulsed EL4 cells than the Hepa1-6^V-NS2′-5B′ cells. The ILDSFDPL-pulsed EL4 cells were not lysed in all groups of mice (**Figure 3d**). Since the cytotoxic effect on Hepa1-6^V-NS2′-5B′ cells revealed activity against unidentified HCV nsPs epitopes, this may mimic the real situation during HCV infection when infected liver cells present multi-epitopes. To determine CTL activities *in vivo*, rSFV prime-boost immunized mice were adoptive-transferred with autologous splenocytes pulsed with HCV peptides and irrelevant peptides 10 days after the boost immunizations. The GAVQNEVTL-pulsed autologous splenocytes were lysed in mice immunized with either rSFVeNS2′-5B′ (90.5% ± 6.7%) or rSFVeNS3/4 (97.9% ± 2.2%) (**Figure 3e**). No cytotoxic effect on the ILDSFDPL-pulsed autologous splenocytes was detected in all groups of mice. Of note, cytotoxic activity was more potent *in vivo* though no extra restimulation of HCV-specific cells was performed. The cytotoxic effector on peptide-pulsed EL4 cells or splenocytes and Hepa1-6 cells expressing all HCV nsPs indicated the potency of these rSFV vaccines inducing functional HCV nsPs–specific CTL *in vivo*.

Therapeutic effect of the rSFV immunizations against EL4 tumor cells

Next, we tested the potencies of these rSFV vaccines to eradicate established HCV nsPs–expressing tumor. Mice subcutaneously inoculated with EL4^V-NS3/4A cells, expressing only HCV NS3 and NS4A, were immunized three times with rSFVeNS2′-5B′, rSFVeNS3/4A or PBS on days 2, 9, and 16 after tumor inoculation. Mice inoculated with EL4^V-NS5A/B′ cells, expressing only HCV NS5A and NS5B, were treated with either rSFVeNS2′-5B′, rSFVeNS5A/B′ or PBS with the same immunization schedule. These EL4 tumor cells express diverse levels of HCV nsPs and VENUS protein mimicking the expression of nsPs by naturally HCV-infected hepatocytes (**Supplementary Figure S4**). With this immunization schedule, the expansion phase of effector T cells after the third immunizations will take place during the exponential growth of tumor cells (days 15–20 after tumor inoculation) as observed in PBS-treated mice. Immunizations with both rSFVeNS2′-5B′ and rSFVeNS3/4A induced delay in growth of EL4^V-NS3/4A cells ($P < 0.001$) (**Figure 4a**). Of note, immunizations with rSFVeNS3/4A synchronized the rate of tumor growth that was not observed in PBS-treated mice (**Supplementary Figure S5a**). An immunization effect on EL4^V-NS5A/B′ cells was undetectable (**Figure 4a** and **Supplementary Figure S5b**).

The effect of immunizations on EL4 tumor cells was further analyzed by analyzing the expression of VENUS protein, being coexpressed with the HCV nsPs. EL4 tumor cells were isolated at different time points when the tumor volume reached ~1 cm^3. VENUS$^+$ cells frequencies were reduced in EL4^V-NS3/4A tumor cells in rSFV-immunized mice but not in PBS control ($P < 0.01$) (**Figure 4b**). VENUS expression levels within the VENUS$^+$ cell population (**Supplementary Figure S5c**) and total EL4^V-NS3/4A cell population (**Supplementary Figure S5d**) was also reduced. This effect was not time-dependent as expression differences between groups of mice were detected in mice that were sacrificed on the same day. There was no reduction of the number of VENUS$^+$ cells (**Figure 4b**) and the expression level of VENUS in VENUS$^+$ cells (**Supplementary Figure S5c**) and total EL4^V-NS5A/B′ tumor cells (**Supplementary Figure S5d**) remained the same in all groups of mice. Immunizations with rSFVeNS2′-5B′ or rSFVeNS3/4A delayed tumor growth by reducing the HCV NS3/4A expression and the number of VENUS$^+$ cells in the tumor mass.

To investigate the effect of T cells during the early formation of tumor, the first rSFV immunization was performed before tumor inoculation. Mice were immunized with 5×10^6 rSFV particles on day −5, followed with EL4^V-NS3/4A cells inoculation on day 0. Mice were then boosted with rSFV particles twice on day 2 and day 9 after tumor inoculation. The effect on the growth of tumor was mild (**Figure 5a**). Nevertheless, immunizations with rSFVeNS2′-5B′ and rSFVeNS3/4A induced reduction on the number of VENUS$^+$ cells (**Figure 5b**) and expression

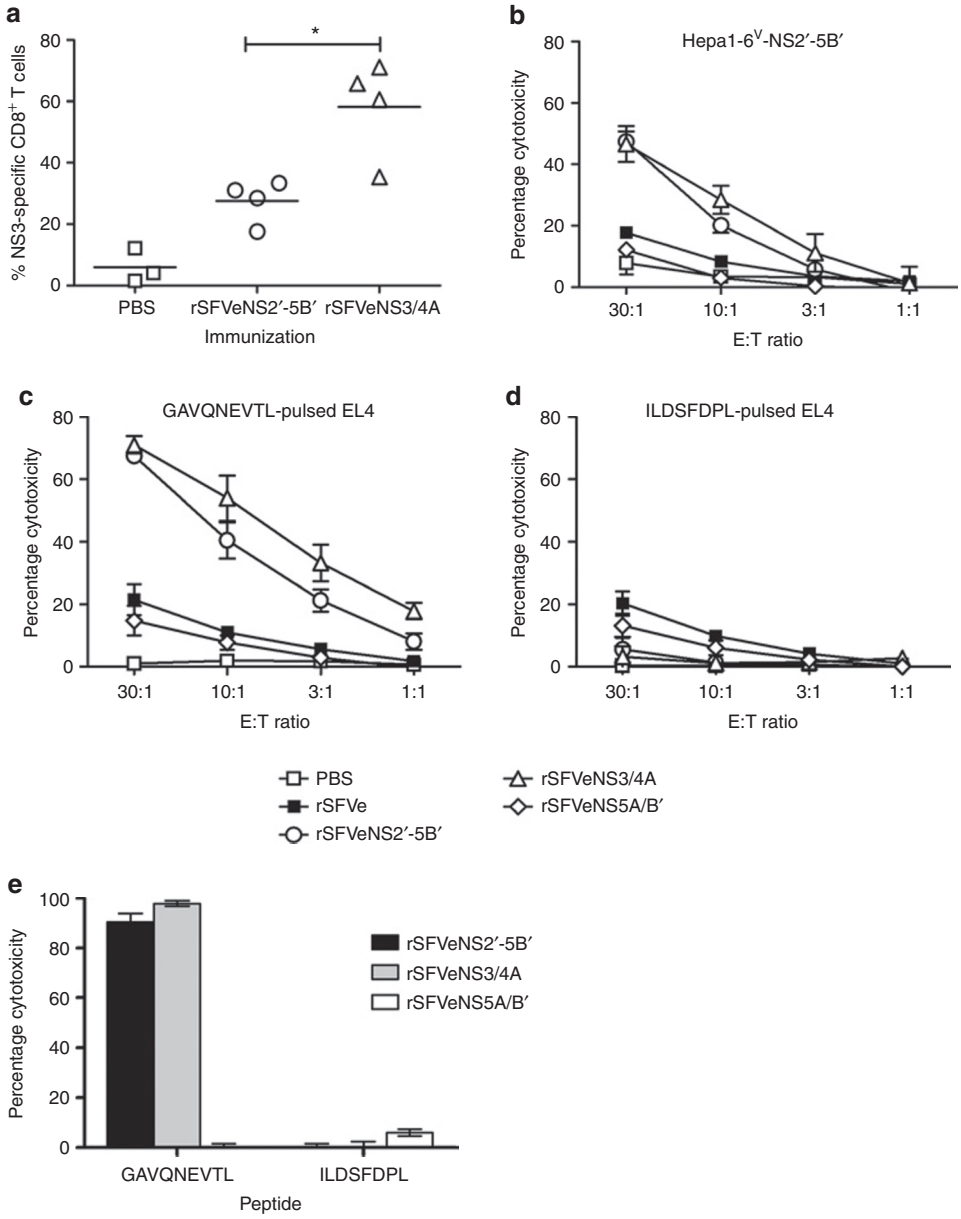

Figure 3 Induction of hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL) with recombinant Semliki Forest virus (rSFV) immunizations. Mice were primed and boosted intramuscularly with 5×10^6 rSFV or phosphate-buffered saline (PBS) with a 2-week interval. Ten days after the last immunization, mice were sacrificed and splenocytes were isolated for *in vitro* bulk CTL assay. Splenocytes were cultured with irradiated Hepa1-6^V-NS2′-5B′ cells at a ratio of 25:1. After a 7-day culture, splenocytes were subjected to (**a**) NS3-dextramer staining and (**b**–**d**) bulk CTL assay. Bulk CTL assay was performed by coculturing effector cells, splenocytes, with ^{51}Chromium pulsed target cells at the indicated E:T ratios for 4 hours. Various target cells, (**b**) Hepa1-6^V-NS2′-5B′ cells, EL4 cells pulsed with either (**c**) GAVQNEVTL or (**d**) ILDSFDPL peptides, were cocultured with restimulated splenocytes. (**e**) For *in vivo* CTL assay, mice were immunized with the same protocol and were intravenously transferred with peptides-pulsed fluorochromes-labeled autologous splenocytes on day 10 after the last immunization. Each mouse received a mixture of autologous splenocytes containing SIINFEKL-pulsed $CFSE^{lo}$-labeled, GAVQNEVTL-pulsed $CFSE^{hi}$-labeled and ILDSFDPL-pulsed TRITC-labeled splenocytes at a 1:1:1 ratio. Mice were sacrificed at 14 hours after adaptive transferred. Splenocytes were isolated and the presences of transferred splenocytes were analyzed using flow cytometry. Data represent (**a**) mean and (**b**–**e**) mean ± SD of two to three independent experiments with $n = 2$–4. *$P < 0.05$.

of VENUS in the $VENUS^+$ cell (**Supplementary Figure S6b**) and total $EL4^V$-NS3/4A cell (**Supplementary Figure S6c**). The immunization effect on $EL4^V$-NS5A/B′ was not determined. In both late and early immunization setups, both rSFVeNS2′-5B′ and rSFVeNS3/4A particles reduced the expression of VENUS and the frequencies of $VENUS^+$ EL4 cells demonstrating the activity of HCV-specific $CD8^+$ cells *in vivo*.

DISCUSSION

Therapeutic vaccination against HCV infection aims to induce robust cellular immunity against conserved HCV proteins leading to eradication of HCV-infected liver cells. Here, we demonstrated that an rSFV-based viral vector vaccine encoding HCV NS3/4A as well as an rSFV vaccine encoding all HCV nsPs induced strong T-cell response against an epitope

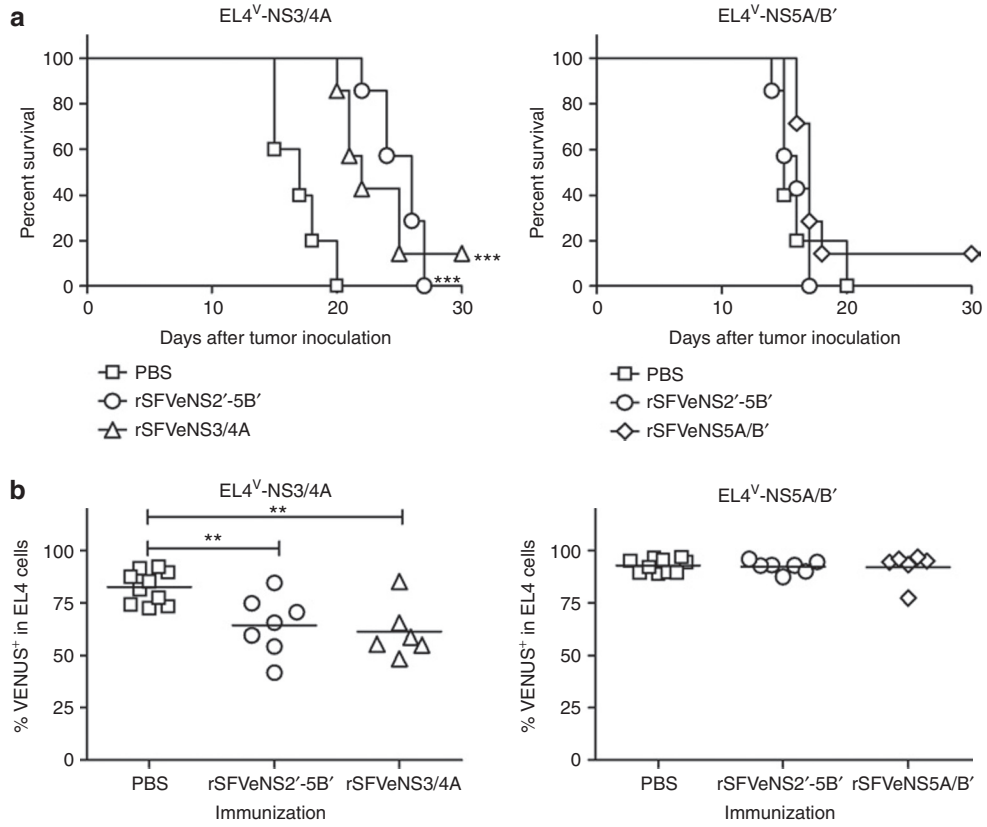

Figure 4 Therapeutic effects of recombinant Semliki Forest virus (rSFV) immunizations against EL4 tumor cells. Naïve mice were subcutaneously inoculated with 5×10^5 of EL4^V-NS3/4A or EL4^V-NS5A/B′ cells. Mice were then immunized intramuscularly with 5×10^6 rSFV on days 2, 9, and 16 after tumor inoculation. (**a**) Survival curve is determined when tumor volume equals to 1 cm^3. (**b**) Mice were sacrificed when the volume of tumor exceeded 1 cm^3. Tumor cells were isolated to analyze the frequency of VENUS$^+$ cells in the EL4^V-NS3/4A and EL4^V-NS5A/B′ cell population using flow cytometry. Cumulative results of two independent experiments with $n = 5–7$ are presented. ***$P < 0.001$, **$P < 0.01$.

within the HCV NS3 protein. This NS3-specific T-cell population consisted of both effector and memory cells. Furthermore, these induced NS3-specific T cells were polyfunctional, secreting multiple proinflammatory Th1 cytokines and possessed cytolytic activity against HCV nsPs–expressing cells *in vitro* and *in vivo*. Immunizations partially protected mice from HCV NS3/4A-expressing tumor formation. Vaccine-induced immune pressure on the tumors lead to selection of tumor cells, resulting in decreased frequencies of HCV NS3/4-expressing EL4 cells and reduced expression levels of the transgene (NS3/4A-VENUS).

To our knowledge, this is the first time showing that an rSFV vector retains its immunogenicity when expressing a foreign transgene as large as 6.1 kb. Previously, it has been shown that rSFV expressing a smaller transgene, HCV NS3/4A, induced NS3-specific CTLs.[17] Due to the limitation of the cellular protein synthesis machinery, increasing the size of the transgene will lower the expression of each protein. As a result, vaccine immunogenicity may be reduced or even lost. We demonstrated that an rSFV vector could be packed with all HCV nsPs, expressing all intact HCV nsPs, and induced a HCV NS3–specific response, although this response was lower than the response induced by rSFV expressing HCV NS3/4A only. Thus, the immunogenicity of rSFV vector is maintained even when a large transgene is inserted. Increasing the size of foreign transgenes allows broadening of the spectrum of T-cell responses which is considered essential for the induction of protective HCV immunity.[18–20]

T-cell responses against $HCV_{2252–2259}$ ILDSFDPL, an epitope recently identified by Holmstrom, F and colleagues,[16] was not detectable this study. It should however be noted that the ILDSFDPL-specific T-cell responses were identified in mice immunized with DNA plasmid expressing NS5A only. Expressing more than one protein may lead to increase competition between epitopes resulting in reduced responses against subdominant epitopes.[21] Yet to allow a non-HLA restricted application of HCV vaccines expression of as many relevant proteins as possible is desirable. Other T-cell epitopes in HCV nsPs were predicted with mathematic algorithms including SYFPEITHI,[22] NetMHCpan 2,8[23] and Immune Epitope Database and Analysis Resource (IEDB).[24] The MHC affinity of the identified peptides was determined and the peptides were used to restimulate splenocytes from rSFVeNS2′-5B′ and rSFVeNS5A/B′ immunized mice. Minor T-cell responses against $NS2_{139–147}$, $NS5B_{2–10}$ and $NS5B_{157–165}$ were observed in both groups of mice (manuscript in preparation). Nevertheless, rSFV-based vaccines encoding all possible T-cell epitopes allow induction of a unique spectrum of immune response depending on the patient's HLA alleles.

The magnitude of a T-cell response is positively correlated with the dominance of its cognate epitope.[25] Activation of T cells against a protective but not pathogenic

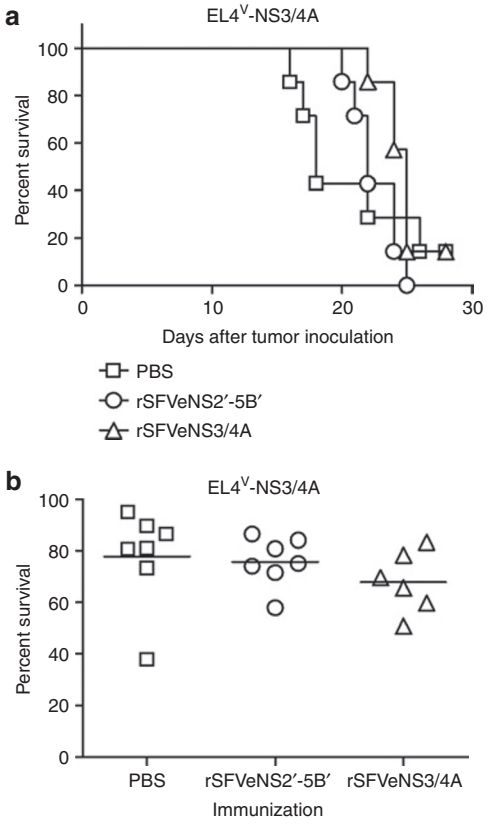

Figure 5 Effect of early immunizations with rSFVeNS2′-5B′ and rSFVeNS3/4A against EL4^{V}-NS3/4A tumor cells. Mice were intramuscularly immunized thrice with 5×10^6 recombinant Semliki Forest virus (rSFV) on days –5, 2, and 9. On day 0, immunized mice were subcutaneously inoculated with 5×10^5 of EL4^{V}-NS3/4A cells. (**a**) Survival curve is determined when tumor volume equals 1 cm^3. (**b**) Mice were sacrificed when the volume of tumor exceeded 1 cm^3. Tumor cells were isolated and the frequencies of VENUS$^+$ cells in the EL4^{V}-NS3/4A cell population were analyzed using flow cytometry. Data represent results from one independent experiments with $n = 7$.

immunodominant epitope results in control of virus-induced diseases.[26] We observed induction of CD8$^+$ T-cell responses against an immunodominant epitope located in the HCV NS3 protein in mice immunized with rSFV expressing either NS3/4A or all HCV nsPs. In the latter group however, lower frequencies were observed. Since the anti-tumor effect of both rSFV vectors was similar, this suggests that T-cell responses against this immunodominant T-cell epitope are protective when a threshold frequency is reached.[27]

On the other hand, activation of T cells against one immunodominant epitope facilitates selection of viral escape mutants.[28,29] Furthermore, enhanced responses against immunodominant epitopes will reduce or even abrogate response against subdominant T-cell epitopes narrowing the T-cell repertoire.[26,30] Therefore, T-cell responses against subdominant epitopes are desirable.[31,32] Interestingly, we observed a decreased response against the immunodominant epitope ($HCV_{1629–1637}$ GAVQNEVTL) in mice immunized with rSFV expressing all HCV nsPs as lower frequencies of GAVQNEVTL-specific T cells were detected which may indeed allow enhanced responses against other subdominant epitopes.[33]

The functionality of *de novo* induced antigen-specific CD8$^+$ cells is also a reliable indicator for effective immunizations.[34] A hallmark of an effective antiviral T cell is the secretion of multiple Th1 cytokines including IFN-γ, TNF-α, and IL-2.[34] IFN-γ and TNF-α mediate control of intracellular infections synergistically[35] while IL-2 strongly enhances the expansion of both CD4$^+$ and CD8$^+$ T cells and the development of memory cells.[36] Furthermore, it has been shown that IFN-γ reduces the expressing of HCV nsPs *in vitro*.[37] In a recent study, heterologous prime-boost immunizations with a DNA and a MVA vaccine successfully enhanced the number of multifunctional T cells.[38] In our study, with homologous prime-boost rSFV immunizations, function-active HCV-specific T cells secreting IL-2, IFN-γ, and TNF-α were induced. In addition, we showed that the HCV-specific T-cell population contained not only effector T cells, but also high numbers of cells with a memory phenotype (both T_{CM} and T_{EM} cells). Central memory T cells have a high proliferation rate and are high producers of IL-2, whereas effector memory cells are potent producers of IFN-γ and cytotoxic granules.[39] Phenotype and cytokine secretion of HCV-specific T cells reflect the potency of rSFV-based vaccines. It would be highly interesting to study the immunogenicity of rSFV vectors in mice or other animal models with exhausted T cells as patients with CHC have dysfunctional T cells.[10,11]

Small animal models susceptible for HCV infection are not available yet, and still under development.[40] To investigate the immunogenicity of our rSFV-based HCV vaccines, we therefore produced a polyclonal EL4 tumor cells line by transducing EL4 with HCV nsPs–expressing lentivirus. These tumor cells are heterogeneous, expressing variable levels of HCV nsPs as indicated by the expression level of the cotransduced reporter gene VENUS. Also, in natural HCV infections, this heterogeneity in expression level occurs because of the HCV genetic diversity within a host.[41–43] Eradication of these heterogenic tumors involved negative selection and lysis of the VENUS^{hi} tumor cells by tumor-specific CD8$^+$ T cells. Furthermore, the kinetics of effector CD8$^+$ T-cell responses plays a crucial role on the developing tumor. In this EL4 model, tumor developed exponentially from days 15 to 25 after tumor inoculation. To investigate the therapeutic efficacy of the rSFV-based HCV vaccines, immunizations were scheduled such that the peak of CD8$^+$ T-cell responses overlapped with the exponential growth of the EL4 tumor. Since immunizations were given after tumor inoculation, time for negative selection of VENUS^{hi} EL4 cells was short (approximately from day 6 to day 15). As a result, eradication of tumor cells occurred during the expansion and plateau phases of T-cell response but the reduction of VENUS expression was moderate. In other immunization setups when immunization was given before tumor inoculation, tumor cells were exposed to activated T cells for longer time (from day 0 to day 15) allowing more selection pressure on VENUS^{hi} cells resulting in a significant reduction of the expression of VENUS. However, selection alone was not enough for eradication of this fast-growing tumor as the VENUS^{lo} cells escaped from the immune responses and started to grow exponentially during the contraction phase of T-cell responses. Therefore, rSFV-based HCV immunizations induced immune pressure on HCV nsPs–expressing cells resulting in lower expression of HCV nsPs. Furthermore, the development of the nsPs-expressing EL4 tumor

was delayed by the presence of the robust HCV-specific T-cell responses.

To conclude, rSFV encoding the entire HCV nsPs is a potent vaccine inducing robust and effective HCV-specific immunity. Immune pressure was exerted on HCV nsPs–expressing tumor cells reducing the expression and frequencies of HCV nsPs–expressing tumor cells. The next step will be to study combinations of therapeutic vaccines with other immune interventions and/or direct acting antiviral drugs. For example, PD1/CTLA-4 blockage[44,45] and anti-TIM3 treatment[46] have been shown to restore HCV-specific dysfunctional T cells *in vitro*, while direct acting antiviral drugs aimed to reduce viral load result in reduced production of viral mutants as well as a decreased T-cell exhaustion.[47] Combined treatment of a potent HCV-specific vaccine with immune interventions and/or direct acting antiviral drugs will hopefully result in *de novo* HCV-specific T cells and restoration of existing dysfunctional T cells resulting in a better prognosis in patients at various stages of HCV infection.

MATERIALS AND METHODS

Cell culture. Baby hamster kidney cells (BHK-21, ATCC #CCL-10) were obtained from the American Type Culture Collection and were maintained in RPMI1640 (Life Technologies, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum (FBS) (Lonza, Basel, Switzerland). EL4 lymphoma cells and RMA-S cells were kindly provided by Cornelis JM Melief (Leiden University Medical Center, The Netherlands) and were maintained in IMDM (Life Technologies) supplemented with 10% FBS. Hepa1-6 cells (provided by Jurgen Seppen, Academic Medical Center, Amsterdam, The Netherlands) and Human embryonic kidney (HEK) 293T cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS. All cells were cultured with 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies) at 37 °C with 5% CO_2 otherwise indicated.

Construction of rSFV replicon vectors. pSFV-helper 2 and pSFV4.2 (pSFV), were provided by P Liljestrom (Karolinska Institute, Stockholm, Sweden). The plasmid DNA containing the full-length cDNA of HCV H77 genotype 1a consensus sequence (H/FL) was kindly provided by Charles M Rice via Apath, LLC (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: p90HCVconsensuslongpU).[48] pUC57-enh which contains the translational enhancer, foot-and-mouth disease virus 2A auto-protease fragment, was synthesized by Eurogentec (Maastricht, the Netherlands). pSFVe (10,764 bps) was generated by subcloning the translational enhancer from pUC57-enh into pSFV between the BamHI and BssHII sites. pSFVeNS2′-5B′ (16,838 bps) was generated by subcloning NS2′-5B′ from H/FL into pSFVe between the BssHII and NotI sites. pSFVeNS3/4A (12,839 bps) was constructed by cloning the BssHII-NS3/4A-SpeI fragment, which was amplified by PCR using H/FL as a template DNA, into pSFVe between the BssHII and SpeI sites. pSFVeNS5A/B′ (13,700 bps) was constructed by cloning the BssHII-NS5A/B′-NotI fragment, which was amplified by PCR using H/FL as a template DNA, into pSFVe between the BssHII and NotI sites. All restriction enzymes were purchased from Thermo Fisher scientific (Landsmeer, The Netherlands). DNA sequences were verified by sequence analysis.

Production, purification and titer determination of rSFV. rSFVe, rSFVeNS2′-5B′, rSFVeNS3/4A, and rSFVeNS5A/B′ were produced as previously described.[49] In brief, the plasmid DNA was *in vitro* transcribed into RNA using SP6 RNA polymerase (GE Healthcare, Diegem, Belgium). RNA of pSFV encoding various parts of HCV nsPs and pSFV-helper 2 were cotransfected at a molar ratio of 1:1 into BHK-21 cells. Likely due to the size of rSFVeNS2′-5B′, lower production of rSFVeNS2′-5B′ was observed. Therefore, transfected BHK-21 cells were cultured at 30 °C with 5% CO_2 for 78 hours instead of 37 °C for 36 hours as described previously. The supernatant containing the viral particles was collected and titered with BHK-21 cells. Titer of unpurified rSFVeNS2′-5B′ was $\sim 2 \times 10^7$ particles/ml and titers of other rSFV particles were $\sim 1 \times 10^8$ particles/ml. rSFV particles were further purified on a discontinuous sucrose density gradient and titrated with BHK-21 cells.

Production of HCV nsPs–expressing lentivirus and HCV nsPs–expressing cell lines. Packaging construct (pCMV 8.91), Glycoprotein envelop plasmid (pMD2.G) and lentiviral vector expressing VENUS (442 New pRRL. PPT.SF.IRES-VENUSSnucmer pre or pLenti) were kindly provided by Jan Jacob Schuringa (University of Groningen, The Netherlands). pLenti-NS2′-5B′-VENUS was generated by subcloning the BglII-NS2′-5B′-BglII fragment from pSFVeNS2′-5B′ into the BamHI site of pLenti vector. pLenti-NS3/4A-VENUS was generated by removing the AgeI-NS5A/B′-Eco81I fragment from pLenti-NS2′-5B′-VENUS. The sticky ends of AgeI and Eco81I were then filled by Klenow fragment and self-ligated. pLenti-NS5A/B′-VENUS was generated by removing Bst1107I-NS2′-4A-Eco81I from pLenti-NS2′-5B′-VENUS. The sticky ends produced by Eco81I were filled in by Klenow fragment and ligated to the blunt ends produced by Bst1107I. To confirm the expression of HCV nsPs, HEK 293T cells were transfected with each plasmid DNA and the expressed nsPs were determined by western blot analysis (data not shown).

Production of lentivirus and transduction of target cells was performed as previously described for other lentiviruses and cells.[50] In brief, HEK 293T cells were transiently transfected using FUGENE HD (Promega, Leiden, The Netherlands) with pCMV 8.91, pMD2.G and pLenti DNA (pLenti-NS2′-5B′-VENUS, pLenti-NS3/4A-VENUS, pLenti-NS5A/B'-VENUS, or pLenti-VENUS) at a molar ratio of 3:0.7:3. HEK 293T culture medium was replaced by complete medium of EL4 cells or Hepa1-6 cells 24 hours after transfection. The supernatant containing lentiviral particles were harvested, passed through a 0.45 µm filter and stored at −80 °C.

Hepa1-6^V (^{V} represents VENUS), Hepa1-6^V-NS2′-5B′, $EL4^V$-NS3/4A, $EL4^V$-NS5A/B′ cells were generated by transducing Hepa1-6 or EL4 cells with corresponding lentivirus particles. Two cycles of transduction were performed with a 6-hour interval. Forty-eight hours after the second transduction, cells were harvested and the $VENUS^+$ population was isolated by cell sorting using MoFlo Astrios (Beckman coulter, Woerden, The Netherlands). Cell sorting was performed thrice with a 1-week interval to produce stable cell lines. Expression of VENUS was verified using FACSCalibur cytometer (BD Bioscience, Breda, The Netherlands). Fluorescence activated cell sorting (FACS) data were analyzed using FlowJo analysis software (Tree Star, Ashland, OR) otherwise indicated. More than 95% of the sorted cells had stable expression of VENUS after 1 month of culture (**Supplementary Figure S1 and S4**). VENUS expression of each transduced cells was verified before every experiment.

Protein expression by pulse labeling. BHK-21 cells (5×10^5 cells in a well of a six-well plate) were incubated with 5×10^6 rSFVe, rSFVeNS2′-5B′, rSFVeNS3/4A or rSFVeNS5A/B′. After 6 hours, supernatant was removed and the cells were washed with PBS three times. The cells were then further cultured in methionine-free DMEM for 30 minutes followed by labelling with ^{35}S-methionine (0.37 Mbq/well) for 1 hour (PerkinElmer, Groningen, The Netherlands). After 1, 6, or 18 hours of [^{35}S]-methionine labeling, cells were washed with cold PBS and then lysed with TENT lysis buffer (50 mmol/l Tris–HCl, 5 mmol/l EDTA, 150 mmol/l NaCl, and 0.5% Triton-X-100, pH7.5) containing 0.2 mmol/l phenyl-methane-sulphonyl-fluoride. Cell lysate was analyzed by SDS/PAGE and autoradiography.

Synthetic peptides. The synthetic peptides $HCV_{1629–1637}$ GAVQNEVTL (H-$2D^b$), $HCV_{2252–2259}$ ILDSFDPL (H-$2K^b$), $OVA_{257–264}$ SIINFEKL (H-$2K^b$) and human papillomavirus-16-$E7_{49–57}$ RAHYNIVTF (H-$2D^b$) were manufactured by the department of Immunohematology, Leiden University

Medical Center, The Netherlands. The purities of the synthetic peptide were analyzed with HPLC. All synthetic peptides have a purity of >90%.

Peptides stabilization assay. RMA-S cells were cultured at 26 °C with 5% CO_2 for 48 hours to induce expression of MHC class I. Cells were then incubated with various concentrations of synthetic peptides at 26 °C for 4 hours, followed by cultured at 37 °C for 1 hour. Cells were harvested, washed once with 0.5% bovine serum albumin/PBS and stained with APC-anti-H-2K^b Ab (clone: AF6-88.5.5.3) and FITC-anti-H-2D^b Ab (clone: 28-14-8) (eBioscience, Vienna, Austria) at 4 °C for 20 minutes. The surface expression of MHC class I molecules were analyzed by FACSCalibur cytometer (BD Bioscience).

Mice. Specific pathogen-free female inbred C57BL/6JOlaHsd (H-2^b) mice were obtained from a commercial vendor (Harlan CPB, Zeist, The Netherlands) and were kept under the institute guidelines of the University of Groningen, The Netherlands. All mice were 8 to 10 weeks of age at the start of all experiments. All animal experiments (DEC number: 5946) were approved by the local Animal Experimentation Ethical Committee (the Institutional Animal Care and Use Committee of the University Medical Center of Groningen).

Prime-boost immunizations. Mice were intramuscularly primed and boosted immunized with a 2-week interval with 5×10^6 rSFV (rSFVe, rSFVeNS2′-5B′, rSFVeNS3/4A, or rSFVeNS5A/B′) in 50 µl (25 µl/thigh muscle) under anesthesia (isoflurane/O_2). For negative controls, the same volume of PBS was injected intramuscularly.

Phenotypic analysis of NS3-specific cells. Splenocytes and blood cells were stained with PE-GAVQNEVTL-dextramers (Immudex, Copenhagen, Denmark) in 5% FBS/PBS for 10 minutes at room temperature. Followed by staining with PE-Cy7-anti-CD8a Ab (clone: 53–6.7), PerCP-Cyanine5.5-anti-CD44 Ab (clone: IM7), APC-anti-CD62L Ab (clone: MEL-14), and eFluor 450-anti-CD127 Ab (clone: A7R34) for 20 minutes at 4 °C. All antibodies were purchased from eBioscience. Death cells were excluded with 4′-6′-diamidino-2-phenylindole staining. FACS analysis was conducted with LSR-II flow cytometer (BD Bioscience).

Identification of multifunctional HCV-specific cells. Splenocytes isolated from immunized mice were cultured with the 100 Gy-irradiated Hepa1-6 cells (Hepa1-6^V-NS2′-5B′ cells or Hepa1-6^V cells) at a ratio of 25:1 or with 10 µg/ml of synthetic peptides. The Hepa1-6 cells were cultured in the presence of 50 U/ml of recombinant mouse IFN-γ (Peprotech, London, UK) for 48 hours before coculturing with splenocytes. Splenocytes were cultured in the presence of anti-CD28 Ab (clone: PV-1, Bioceros B.V., Utrecht, The Netherlands), eFluor 660-anti-CD107a Ab (clone: eBio1D4B) and eFluor 660-anti-CD107b Ab (clone: eBioABL-93) in a 96-well plate at 37 °C with 5% CO_2. One hour after culture, brefeldin A (1 mg/ml) was added and the cultures were further incubated for 4 hours. Cells were then harvested, washed and stained with LIVE/DEAD fixable violet dead cell stain kit (Life Technologies) according to manufacturer's manuals. Followed by surface staining with PE-Cy7-anti-CD8a Ab at 4 °C for 20 minutes and intracellular staining with PerCP-Cyanine5.5-anti-IFN-γ Ab (clone: XMG1.2), FITC-anti-TNF-α Ab (clone: MP6-XT22) and APC-Cy7-IL-2 Ab (clone: JES6-5H4) at 4 °C for 30 minutes. eFluor 660-anti-CD107a, eFluor 660-anti-CD107b and PerCP-Cyanine5.5-anti-IFN-γ antibodies were purchased from eBioscience; FITC-anti-TNF-α and APC-Cy7-IL-2 antibodies were purchased from BD Biosciences. FACS analysis was conducted with LSR-II flow cytometer, and data were analyzed using FCOM tool of WinList software (Verity Software House, Topsham, ME) and presented as pie charts using SPICE version 5.3 (NIAI freeware).

Bulk CTL assay. The stimulator cells, Hepa1-6^V-NS2′-5B′ cells, were cultured in the presence of 50 U/ml recombinant murine IFN-γ for 48 hours. Hepa1-6^V-NS2′-5B′ cells were then irradiated (100 Gy) and cocultured with effector cells, splenocytes, at a ratio of 1:25 in a T25 flask at 37 °C with 5% CO_2. Recombinant human IL-2 (5 U/ml) (Peprotech) was added on day 3 and day 5 of the culture. After 7 days culture, splenocytes were harvested and cocultured with ^{51}Chromium (^{51}Cr)-labeled target cells (Hepa1-6^V-NS2′-5B′ cells, EL4 pulsed with GAVQNEVTL or EL4 pulsed with ILDSFDPL). Target cells were cultured in the presence of recombinant murine IFN-γ (50 U/ml) 48 hours before coculture with effector cells. Target cells were labeled with ^{51}Cr (100 µCi/2×10^6 cells) (PerkinElmer) in the presence or absence of synthetic peptides (10 µg/ml) for 1 hour at 37 °C. Coculture of effector cells and target cells were performed in 96 well plate at 4 E:T ratios in triplicates at 37 °C with 5% CO_2. After 4 hours of culture, supernatants were harvested and analyzed with a RiaStar manual gamma counter (Packard, Meriden, CT). The percentage of cytotoxicity was calculated according to the formula: % specific release = ((experimental release − spontaneous release)/(maximal release − spontaneous release)) count per minute (c.p.m).

In vivo *CTL assay.* Splenocytes were isolated from naïve syngeneic mice and were pulsed with synthetic peptides at 10 µg/ml in IMDM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin for 2 hours at 37 °C with 5% CO_2. The peptide-pulsed splenocytes were then washed and resuspended in PBS. GAVQNEVTL-pulsed and SIINFEKL-pulsed splenocytes were labeled with 5 µmol/l of CFSE (Life Technologies) and 0.1 µmol/l of CFSE for 20 minutes at 37 °C, respectively. ILDSFDPL-pulsed splenocytes were labeled with 30 µg/ml of tetramethylrhodamine-6-isothiocyanate (TRITC) (Life Technologies) for 10 minutes at 37 °C. Differentially labeled cells were washed, resuspended in PBS and combined at a 1:1:1 ratio for intravenous adoptive transfer. Each mouse received 2×10^6 cells of each population. After 14 hours, splenocytes were isolated and analyzed using LSR-II flow cytometer. The percentage of cytotoxicity was calculated with the formula: % cytotoxicity = 1 − (% of relevant peptides-pulsed target (GAVQNEVTL or ILDSFDPL) in rSFV-immunized mice/% of irrelevant peptides-pulsed target (SIINFEKL) in immunized mice)/(% of relevant peptides-pulsed target in PBS-treated mice/% of irrelevant peptides-pulsed target in PBS-treated mice).

Tumor inoculation. Mice were inoculated subcutaneously in their right flank with 5×10^5 EL4^V-NS3/4A or EL4^V-NS5A/B′ cells suspended in 0.2 ml PBS. The volume of tumor was measured by caliper. Cylinder tumor is calculated with the formula 0.7854 × width^2 × length (cm^3). Round tumor is calculated with the formula 0.5236 × diameter^3 (cm^3). Mice were euthanized when the tumor volume reached 1 cm^3.

Tumor cells isolation. Tumor excised from mice was cut into small pieces and suspended in 5 ml of William's E + Glutamax medium (Life Technologies) containing 1 mg/ml of collagenase A (Roche Applied Science, Almere, The Netherlands). Tumor suspension was transferred into a gentleMACS C-tube and homogenized with the program "m_imp-TUMOR4" with a gentleMACS dissociator (Miltenyi Biotec, Leiden, The Netherlands) followed by 30 minutes incubation at 37 °C. Homogenization and incubation were performed twice. After the second incubation, cell suspension was passed through a 70-µm strainer (BD Bioscience), wash once with 0.5% bovine serum albumin/PBS and analyzed with FACSCalibur cytometer.

Statistical analysis. Differences between two groups were determined with the Mann–Whitney *U*–test. Differences between two survival curves were calculated using the log-rank (Mantel-Cox) test. All data were analyzed with GraphPad Prism software (La Jolla, CA). $P < 0.05$ was considered statistically significant.

SUPPLEMENTARY MATERIAL

Figure S1. VENUS expression of HCV nsPs–expressing Hepa1-6 cell lines.

Figure S2. Stabilization of MHC class I molecules on RMA-S cells through binding of HCV peptides.

Figure S3. No induction of polyfunctional HCV-specific T cells upon stimulation with Hepa1-6^{V} cells.
Figure S4. VENUS expression of HCV nsPs–expressing EL4 cell lines.
Figure S5. Therapeutic effect of rSFV immunizations against HCV nsPs–expressing EL4 cells.
Figure S6. Effect of early immunization with rSFVeNS2′-5B′ and rSFVeNS3/4A against EL4^{V}-NS3/4A cells.

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